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Indoxyl sulfate impairs valsartan-induced neovascularization☆

Ko-Lin Kuo[a,b], Jin-Feng Zhao[c], Po-Hsun Huang[d,e,f], Bei-Chia Guo[g], Der-Cherng Tarng[d,h,i,*], Tzong-Shyuan Lee[g,**]

[a] Division of Nephrology, Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan
[b] School of Medicine, Buddhist Tzu Chi University, Hualien, Taiwan
[c] MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, Dundee, UK
[d] Institutes of Clinical Medicine, Taipei, Taiwan
[e] Cardiovascular Research Center, School of Medicine, National Yang-Ming University, Taipei, Taiwan
[f] Divisions of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
[g] Graduate Institute and Department of Physiology, College of Medicine, National Taiwan University, Taipei, 10051, Taiwan
[h] Department and Institute of Physiology, National Yang-Ming University, Taipei, Taiwan
[i] Division of Nephrology, Department of Medicine and Immunology Research Centre, Taipei Veterans General Hospital, Taipei, Taiwan

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ABSTRACT

Studies revealed that the use of renin-angiotensin-aldosterone system antagonism is not associated with a statistically significant reduction in the risk of cardiovascular events in patients with chronic kidney disease (CKD) compared with that in the general population. We tested the hypothesis that indoxyl sulfate (IS) can interfere with the protective effect of valsartan-mediated on endothelial function *in vitro* and neovascularization in mice underwent subtotal nephrectomy. In human aortic endothelial cells, we first demonstrated that IS impaired the valsartan-mediated phosphorylation of $eNOS^{Thr495}$, nitric oxide production and tube formation via NADPH oxidase (NOX) and protein kinase C (PKC) phosphorylation, but this effect was suppressed by cotreatment with apocynin and calphostin C. *In vivo*, IS attenuated valsartan-induced angiogenesis in Matrigel plugs in mice. Moreover, in subtotal nephrectomy mice who underwent hindlimb ischemic surgery, valsartan significantly increased the mobilization of endothelial progenitor cells in circulation as well as the reperfusion of blood flow and density of $CD31^+$ capillaries in ischemic limbs. However, IS attenuated the protective effect of valsartan-induced neovascularization and increased the expression of $p\text{-}PKC\alpha^{Ser657}$ and $p\text{-}eNOS^{Thr497}$ in ischemic limbs. Cotreatment of apocynin and calphostin C reversed the IS impaired-neovascularization and decreased the expression of $p\text{-}PKC\alpha^{Ser657}$ and $p\text{-}eNOS^{Thr497}$ in ischemic limbs. Our study suggests that the NOX/PKC/eNOS signaling pathway plays a pivotal role in the IS-mediated inhibition of valsartan-conferred beneficial effects on endothelial function *in vitro* and neovascularization in subtotal nephrectomy mice. We proposed a novel causative role for IS in cardiovascular complications in CKD patients.

1. Introduction

Several studies have demonstrated that a substantially high risk of cardiovascular (CV) events exists in CKD patients [1,2]. Cardiovascular disease (CVD) is the leading cause of death in patients with CKD, especially for those on long-term dialysis [3]. Risk factors for CVD among CKD patients are not fully understood but probably include both conventional and uremia-associated risk factors. Uremic toxins are retained in the circulation of patients with CKD and are classified into free water-soluble low-molecular weight solutes, middle molecules, and

Abbreviations: ARB, angiotensin II receptor blocker; CKD, chronic kidney disease; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; IS, indoxyl sulfate; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; PKC, protein kinase C; ROS, reactive oxygen species; SNx, subtotal nephrectomy

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* Corresponding author. Department and Institute of Physiology and Institute of Clinical Medicine, National Yang-Ming University, Taipei, and Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, 201, Section 2, Shih-Pai Road, Taipei, 11217, Taiwan.
** Corresponding author. Graduate Institute and Department of Physiology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan.
E-mail addresses: dctarng@vghtpe.gov.tw (D.-C. Tarng), ntutslee@ntu.edu.tw (T.-S. Lee).

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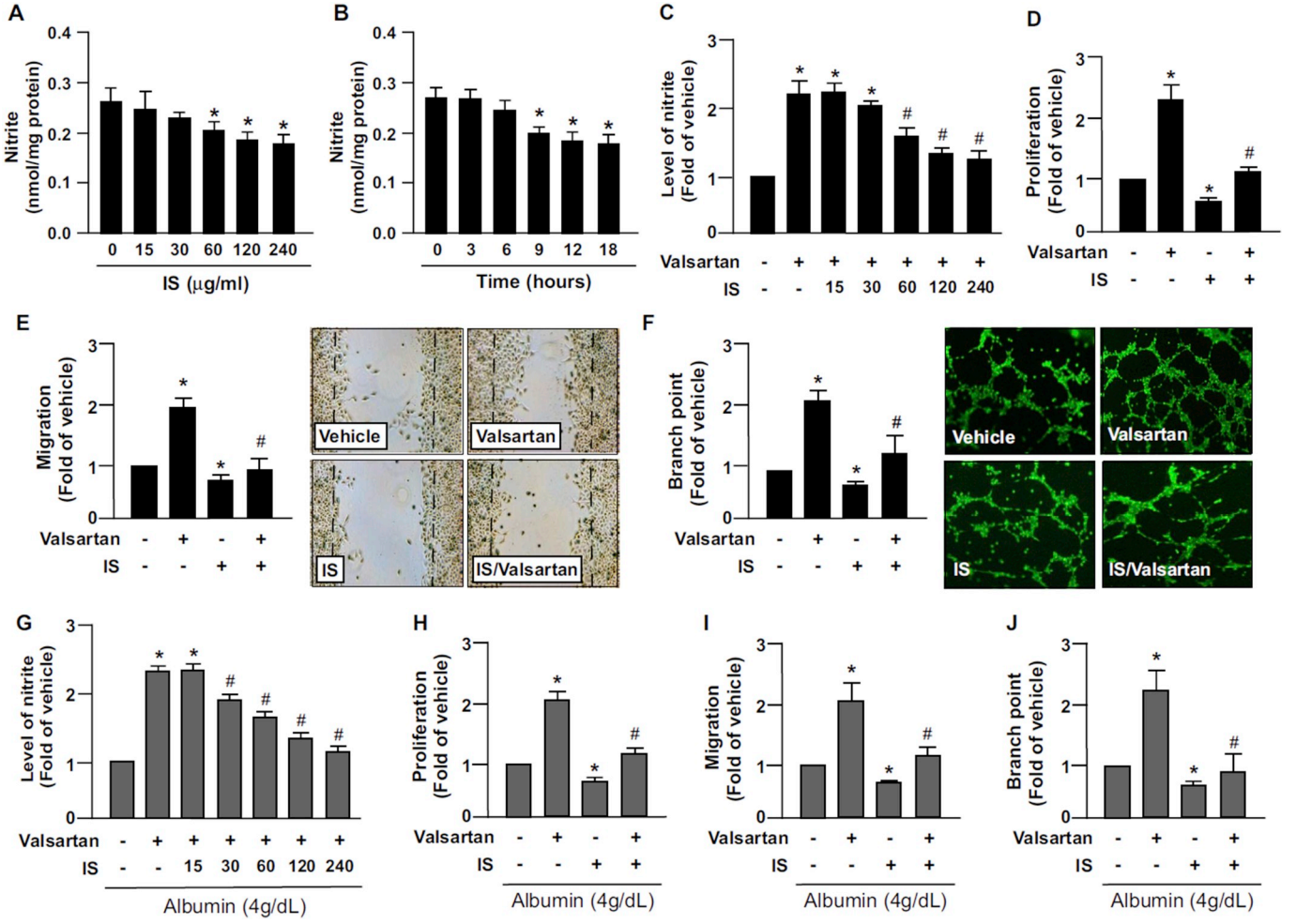

Fig. 1. IS impairs NO generation and attenuates valsartan-mediated NO production and tube formation in HAECs. (A) HAECs were treated with IS (0, 15, 30, 60, 120, 240 μg/mL) for 18 h. (B) HAECs were treated with 240 μg/mL IS for the indicated times (0, 3, 6, 9, 12, 18 h). The level of nitrite in the culture medium was measured by the Griess assay. (C) HAECs were pretreated with IS (15, 30, 60, 120, 240 μg/mL) for 1 h and then with valsartan (10 μM) for 18 h. (D) HAEC proliferation or (E) migration was determined by BrdU incorporation assay or wound healing migration assay, respectively. (F) HAECs were cultured in precoated ECL Cell Attachment Matrix with the indicated treatment agents. Tube formation was visualized; the bar graphs indicate the fold of branch points in 5 randomly selected microscopy views. (G–J) HAECs were pretreated with indicated concentrations of IS for 1 h in the presence of human serum albumin (4 g/dL) and then with valsartan (10 μM) for 18 h. (G) Nitrite level in the culture medium, (H) proliferation, (I) migration, and (J) tube formation were determined. Values are presented as the mean ± SEM of 5 separate experiments. $^{*}P < 0.05$ vs. vehicle-treated cells, $^{\#}P < 0.05$ vs. valsartan-treated cells.

protein-bound solutes, including indoxyl sulfate (IS), indole-3-acetic acid, p-cresyl sulfate, hippuric acid, and homocysteine [4]. Among these protein-bound uremic toxins, IS is the most representative, as a growing number of publications support its biological effects. A number of mechanisms have been reported regarding the detrimental effects of IS accumulation, such as increased reactive oxygen species (ROS) production, upregulation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), and reduced nitric oxide (NO) bioavailability [5–7]. However, conventional dialysis cannot remove IS, as 90% of it is bound to albumin, and IS–albumin complexes are too large to pass through the pores in dialysis membranes. These factors may be underlie why high serum IS levels are associated with a high number of CV events in CKD, even after dialysis [8].

Renin-angiotensin-aldosterone system (RAAS) blockades, including the angiotensin converting enzyme inhibitor (ACE inhibitor) and angiotensin II receptor blocker (ARB), represent the premiere medical therapeutic strategy for combating CKD progression, as heightened angiotensin II is thought to promote vasoconstriction, proteinuria, cellular adhesion, proliferation, and hypertrophy as well as extracellular matrix dysregulation [9–11]. Many of these same processes that contribute to atherosclerosis have been linked to angiotensin II actions [12,13]. Although the cardioprotective effects of RAAS blockades are well established in randomized controlled trials on the general population [14–16], their effects on clinical endpoints in CKD patients remain uncertain [16]. According to a meta-analysis to examine the effect of RAAS blockades on CV outcomes in CKD, RAAS blockades were not associated with a statistically significant reduction in the risk of fatal and nonfatal CV events [17,18]. These studies suggested that uremic toxins may play a pivotal role in interfering with the protective effects of RAAS blockades in CKD.

Endothelial dysfunction has been considered the key step in the pathogenesis of atherosclerosis [19]. Endothelium-derived NO is a critical regulator of the physiological functions of endothelial cells. The production of NO in endothelial cells is generated mainly via the activation of endothelial nitric oxide synthase (eNOS) [20]. Deregulation of eNOS activity is a key event in the development of CVDs, such as atherosclerosis and hypertension [21,22]. Our previous study demonstrated that valsartan, an antagonist of the selective angiotensin II type 1 receptor (AT_1R), rapidly induces the phosphorylation of eNOS at Ser1179 and not at Thr495, accompanied by dissociation of eNOS and AT_1R, which consequently increases NO production via the Src/PI3K/Akt dependent mechanism [23]. Until now, the direct cross-link

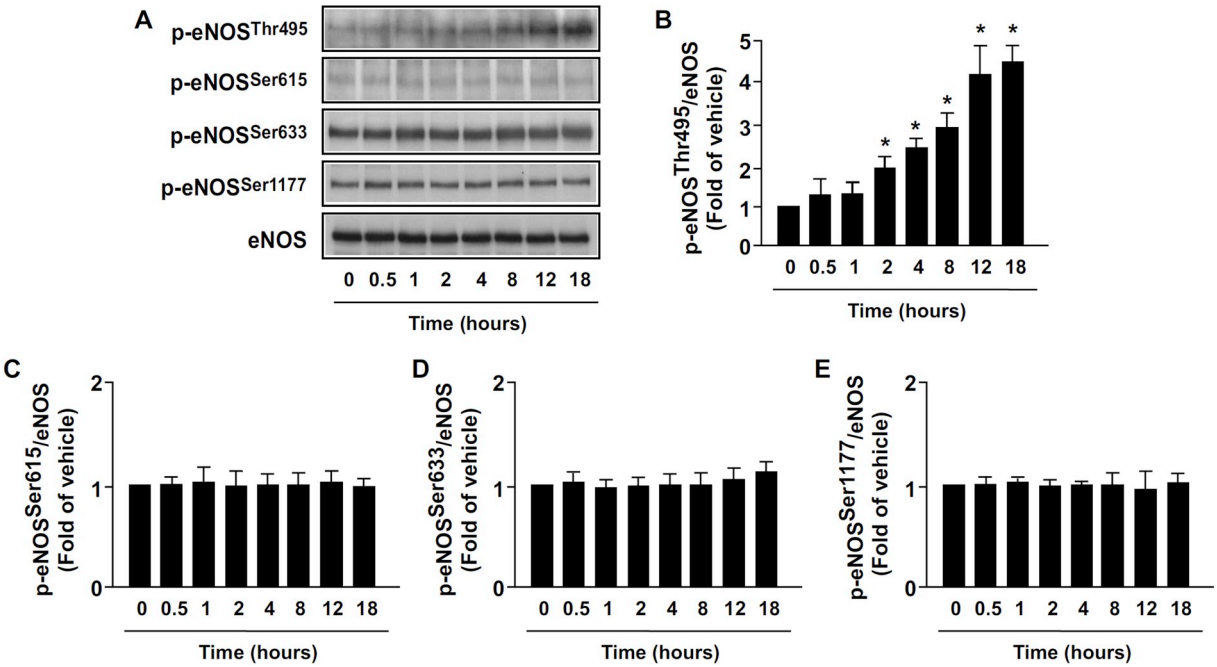

Fig. 2. Effects of IS on eNOS phosphorylation at various sites in HAECs. HAECs were treated with IS (240 μg/mL) for the indicated times (0, 0.5, 1, 2, 4, 8, 12, 18 h). (A–E) Cellular lysates were subjected to Western blot analysis to evaluate the phosphorylation of eNOS at Thr495, Ser615, Ser633, Ser1177 or eNOS. Data are expressed as the mean ± SEM. *$P < 0.05$ vs. vehicle-treated cells.

between IS and ARB signaling in eNOS activity and CV tissues has never been reported. Since severe endothelial dysfunction is frequently observed in CKD [24,25], it is reasonable to postulate that uremic toxins not only direct injury to the endothelium but also interfere with the cardioprotective effect of ARB in CKD.

Therefore, first, we conducted an *in vitro* assay to investigate NO production and various phosphorylation sites and possible signaling cascades in IS and/or valsartan-treated human aortic endothelial cells (HAECs). Second, to confirm our *in vitro* findings, we carried out the Matrigel plug assay to assess the effect of IS on valsartan-elicited angiogenesis *in vivo*. Finally, to mimic the clinical situation in CKD, we explored the effect of IS on valsartan-induced neovascularization and possible mechanisms in subtotal nephrectomy (SNx) mice after hindlimb ischemia (HI) surgery.

2. Materials and methods

A detailed description of the methods can be found in the Supplemental Material.

2.1. In vitro studies

HAECs (Cascade Biologics, Portland, OR, USA) were treated with IS, valsartan, calphostin C, or apocynin. Nitrite was measured by Griess reagent [23]. The membrane-permeable probe hydroethidine (HE) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, Eugene OR, USA) were used to assess intracellular ROS levels [26]. The activity of NADPH oxidase was analyzed using an EnzyChrom™ $NADP^+$/NADPH assay kit [26]. Western blot analysis was performed as described [23]. The cell proliferation, migration and tube formation assay were performed to evaluate endothelial function as previously described [27].

2.2. In vivo studies

The Matrigel plug angiogenesis assay was used to evaluate the formation of new blood vessels *in vivo* as previously described [28]. SNx was induced in 8-week-old male C57BL/6 mice using a 2-step surgical nephrectomy as previously described [27,29]. Five weeks after SNx, hindlimb ischemic surgery was performed on all SNx mice. For SNx, a fluorescence-activated cell sorting (FACS) Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to assess EPC mobilization [28]. The blood flow ratio of the ischemic limb (right)/non-ischemic limb (left) was measured with a laser Doppler perfusion imager system (Moor Instruments Limited, Devon, UK) [28].

2.3. Statistical analysis

The experiments were repeated five times and three replicates were performed in each cell culture experiments. The results are expressed as the mean ± SEM. The Mann-Whitney *U* test was used to compare 2 independent groups. The Kruskal-Wallis test followed by Bonferroni post hoc analysis was used for multiple testing. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. IS attenuates NO production and further inhibits valsartan-induced NO production and tube formation in HAECs

To explore whether IS is capable of inhibiting NO production in HAECs, HAECs were treated with various concentrations of IS or at indicated times, and nitrite levels were assessed. We first found that IS significantly suppressed the nitrite level in a dose-dependent manner at IS concentrations of 60–240 μg/mL (Fig. 1A). IS concentrations of 60–240 μg/mL fall within a higher but clinically achievable range of plasma IS concentrations in dialysis patients [29]. In addition, HAECs were treated with 240 μg/mL for 0, 3, 6, 9, 12, or 18 h. We also found that IS decreased the nitrite levels in a time-dependent manner at 9, 12, and 18 h (Fig. 1B). Our previous study demonstrated that valsartan induced NO production and tube formation in endothelial cells [23]. The effects of IS on valsartan-mediated NO production and tube formation were further examined. HAECs were pretreated with IS concentrations of 15, 30, 60, 120, or 240 μg/mL for 1 h and then with

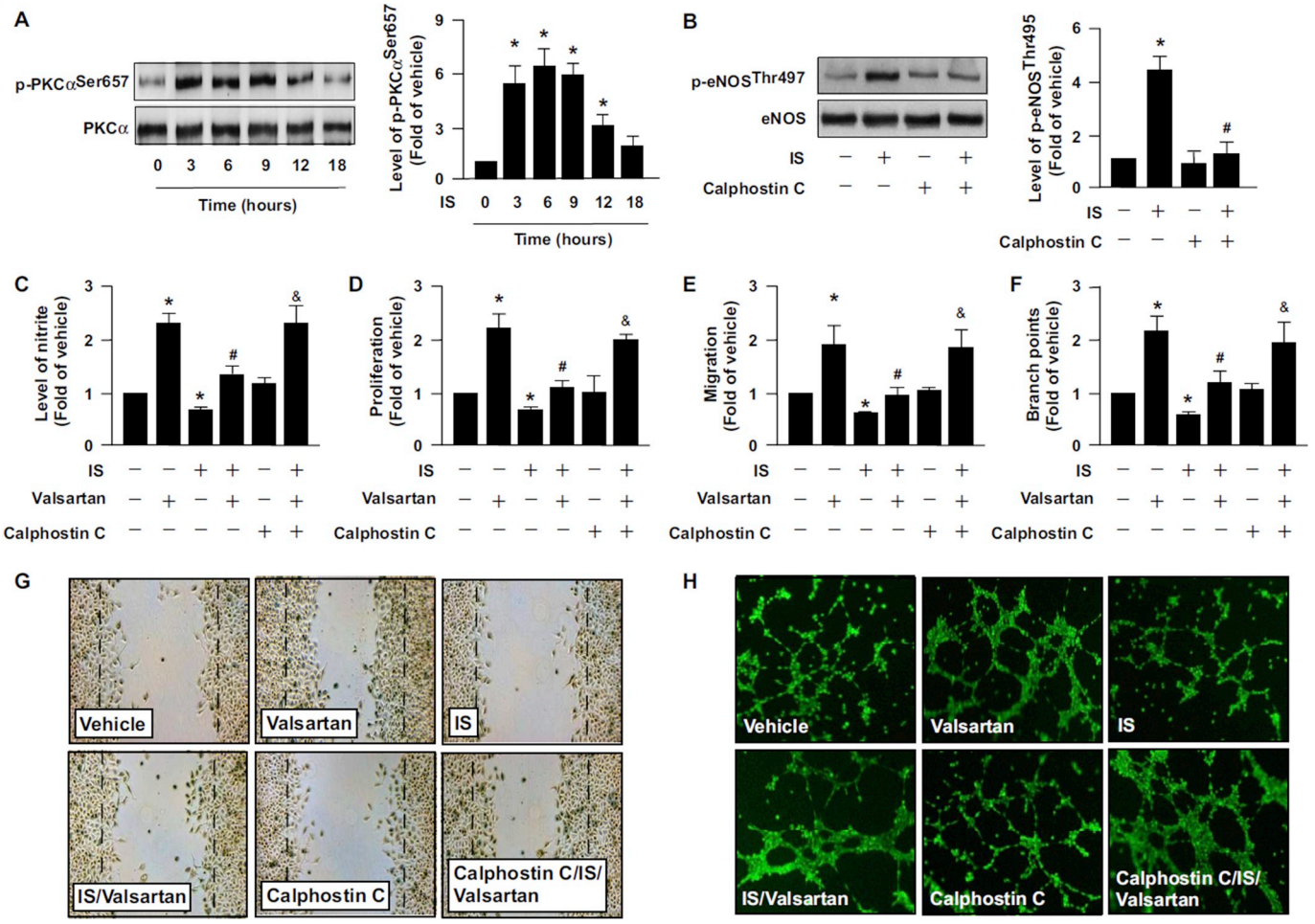

Fig. 3. Effect of IS on PKC phosphorylation, valsartan-mediated NO production and tube formation in HAECs. (A) HAECs were treated with IS (240 μg/mL) for the indicated times (0, 3, 6, 9, 12, 18 h). (B) Cells were preincubated with the PKC inhibitor calphostin C (1 μM) for 2 h and then with IS (240 μg/mL) for 18 h. Cellular lysates were subjected to Western blot analysis to evaluate the phosphorylation of PKCα^{Ser657} and eNOS^{Thr495}. HAECs were incubated with the indicated treatment agent and (C) the level of nitrite in the culture medium, (D) proliferation, (E) migration and (F) tube formation were assessed. (G) The representative images for cell migration. (H) The representative images for tube formation. Data are expressed as the mean ± SEM. $^{*}P < 0.05$ vs. vehicle-treated cells. $^{\#}P < 0.05$ vs. valsartan-treated cells, $^{\&}P < 0.05$ vs. IS + valsartan-treated cells.

valsartan at 10 μM for 18 h. Valsartan significantly increased the nitrite level in HAECs in the absence of human serum albumin (Fig. 1C). Furthermore, IS at concentrations of 60–240 μg/mL significantly decreased nitrite production in HAECs in a dose-dependent manner (Fig. 1C). Similarly, valsartan significantly increased proliferation and migration and improved tube formation in HAECs (Fig. 1D–F). Pretreatment with IS (240 μg/mL) markedly impaired valsartan-induced proliferation and migration and tube formation (Fig. 1D–F). Similarly, IS exhibited a similar inhibitory effect on valsartan-induced NO production, EC proliferation, migration and tube formation in the presence of human serum albumin (4 g/dL) in culture medium (Fig. 1G–J).

3.2. IS increases the phosphorylation of eNOS at Thr495 in HAECs

It is well established that the phosphorylation status of eNOS is critical for the regulation of its activity [27]. Therefore, we assessed the effects of IS on eNOS phosphorylation at various sites. Treatment with IS time-dependently increased the phosphorylation of eNOS at Thr495. However, IS had no effect on the phosphorylation of eNOS at Ser615, Ser633 or Ser1177. These findings suggest that IS may elicit a negative regulatory mechanism to reduce the enzymatic activity of eNOS (Fig. 2).

3.3. IS attenuates valsartan-mediated increase in NO production, proliferation, migration and tube formation in HAECs via increasing phosphorylation of protein kinase C

We next explored the molecular mechanism by which IS impairs NO production. Protein kinase C (PKC) is reportedly the key kinase responsible for the phosphorylation of eNOS at Thr495 in endothelial cells [27]. Our results showed that IS time-dependently increased PKCα phosphorylation at Ser657, which peaked at 3–9 h and then gradually decreased to the basal level (Fig. 3A). Blocking PKC activation by calphostin C diminished the IS-induced phosphorylation of eNOS at Thr495 (Fig. 3B). Additionally, the reduction in valsartan-stimulated NO production, proliferation, migration and tube formation by IS was reversed by pretreatment with calphostin C (Fig. 3C–H). These findings indicate the essential role of the PKC signaling pathway in the inhibitory effects of IS on valsartan-mediated endothelial functions.

3.4. The NADPH oxidase-ROS pathway is required for the detrimental effects of IS on valsartan-mediated endothelial functions

Our data demonstrated that treatment with IS time-dependently increased ROS production and NADPH oxidase activity (Fig. 4A–C). Moreover, the inhibition of NADPH oxidase by apocynin abolished the detrimental effects of IS on ROS production, the phosphorylation of

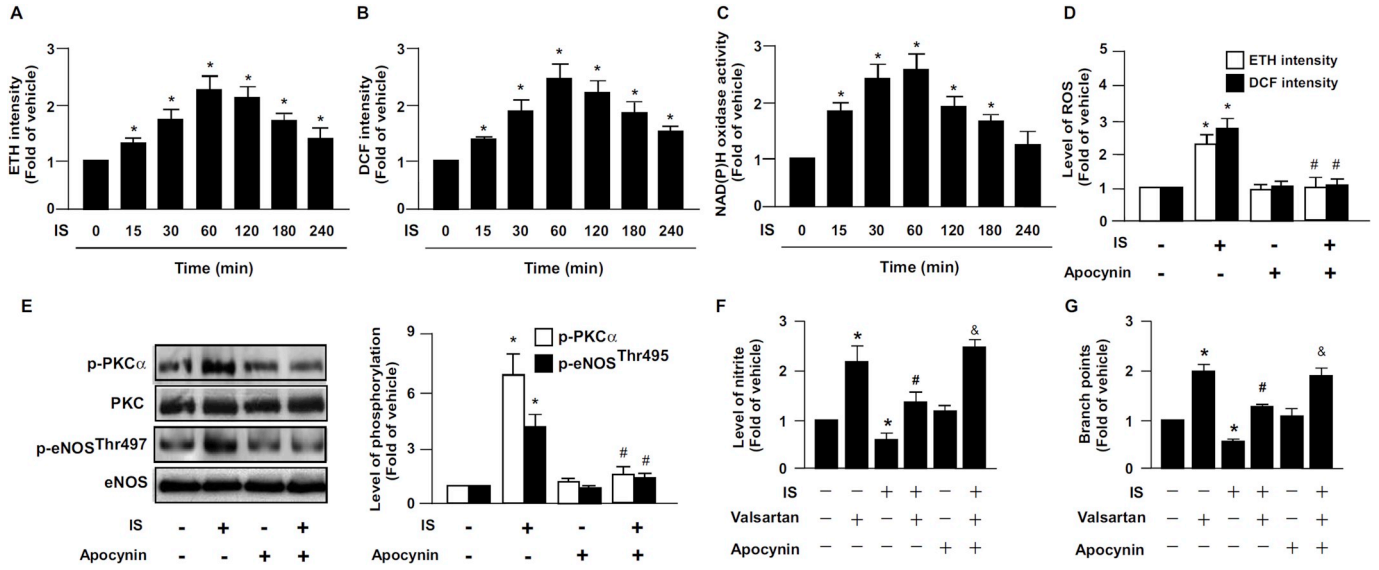

Fig. 4. The NADPH oxidase-ROS pathway plays a crucial role in the harmful effect of IS on valsartan-mediated NO production and tube formation in HAECs. (A–C) HAECs were treated with IS (240 μg/mL) for the indicated times. The membrane-permeable probe hydroethidine and 2′,7′-dichlorofluorescin diacetate were used to examine intracellular ROS levels. (D) HAECs were pretreated with or without the NAD(P)H oxidase inhibitor apocynin (150 μM) for 2 h and then with IS (240 μg/mL) for 60 min. Intracellular ROS levels and NADPH oxidase activities were analyzed by the intensities of the red fluorescent ethidium (ETH) product and the green fluorescent product dichlorofluorescin (DCF) and the $NADP^+$/NADPH assay kit, respectively. (E) Cells were preincubated with apocynin (150 μM) for 2 h and then with IS (240 μg/mL) for 9 h or 18 h. Cellular lysates were subjected to Western blot analysis to evaluate the phosphorylation of PKCα and $eNOS^{Thr495}$. Cells were incubated with the indicated treatment agent and (F) the levels of nitrite in the culture medium and (G) tube formation was assessed. Data are expressed as the mean ± SEM. $^*P < 0.05$ vs. vehicle-treated cells. $^{\#}P < 0.05$ vs. IS-treated cells, $^{\&}P < 0.05$ vs. IS + valsartan-treated cells.

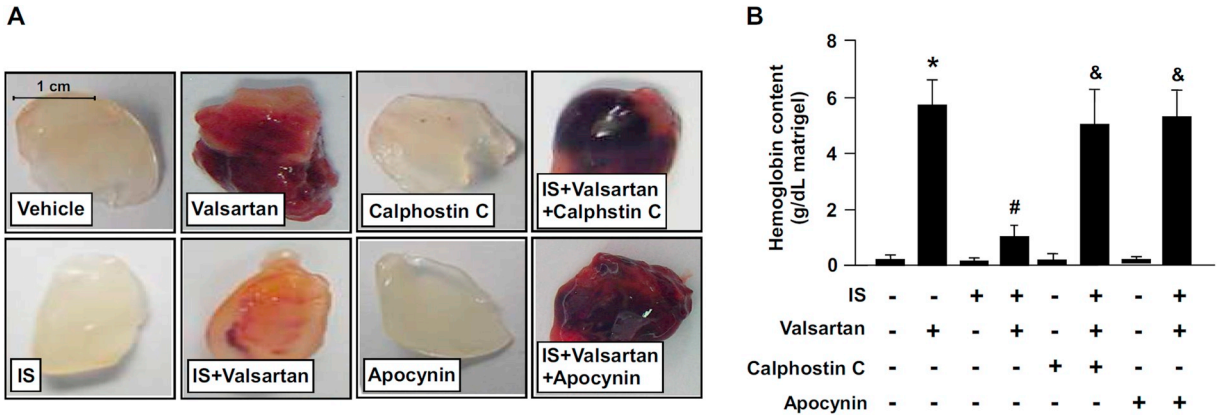

Fig. 5. IS impairs valsartan-induced angiogenesis in *vivo*. Eight-week-old male wild-type mice were subcutaneously injected with Matrigel plugs containing the indicated treatment agents. At 7 days post administration, the plugs were removed and photographed, and the hemoglobin content was analyzed. Data are presented as the mean ± SD from 10 mice. $^*P < 0.05$ vs. vehicle-treated mice. $^{\#}P < 0.05$ vs. valsartan-treated mice, $^{\&}P < 0.05$ vs. valsartan + IS-treated mice.

PKCα and eNOS at Thr495, and the valsartan-mediated NO production and tube formation (Fig. 4D–G). These results suggest that the NADPH oxidase-ROS pathway plays an important role in the inhibitory effects of IS on valsartan-mediated endothelial functions.

3.5. IS limits valsartan-elicited angiogenesis in vivo

To confirm the *in vitro* findings, we used Matrigel plug assays to assess the effects of IS on valsartan-elicited angiogenesis *in vivo*. As shown in Fig. 5, valsartan promoted vascularization, as determined by the hemoglobin content, in Matrigel plugs in wild-type mice. Combined treatment of the Matrigel plugs with IS and valsartan significantly decreased the content of hemoglobin compared with that in the group treated with only valsartan. Moreover, the limitations of IS on angiogenesis were markedly restored by cotreatment with calphostin C or apocynin. These findings suggest that the NADPH oxidase-ROS-PKC signaling pathway plays a pivotal role in the adverse effects of IS-mediated valsartan inhibition on endothelial function *in vivo*.

3.6. IS impairs valsartan-mediated blood perfusion in the ischemic limbs of SNx mice after HI surgery

To mimic the clinical situation in CKD, we explored the effect of IS on valsartan-induced neovascularization and possible mechanism in SNx mice after HI surgery. Fig. 6A showed the protocol of the effect of IS on valsartan-induced neovascularization in SNx mice after HI surgery. The plasma IS and blood pressure levels in each SNx mouse group were shown in Fig. S1 and Fig. S2, respectively. The plasma IS level was similar to the current average value of 23.1 μg/mL observed in an average dialysis population as reported in the most recent review of the European Uremic Toxin Group [30]. Moreover, valsartan significantly improved the blood flow reperfusion in the ischemic limbs of the SNx mice. However, IS impaired the protective effect of valsartan-induced increase in blood perfusion in ischemic limbs. Cotreatment with

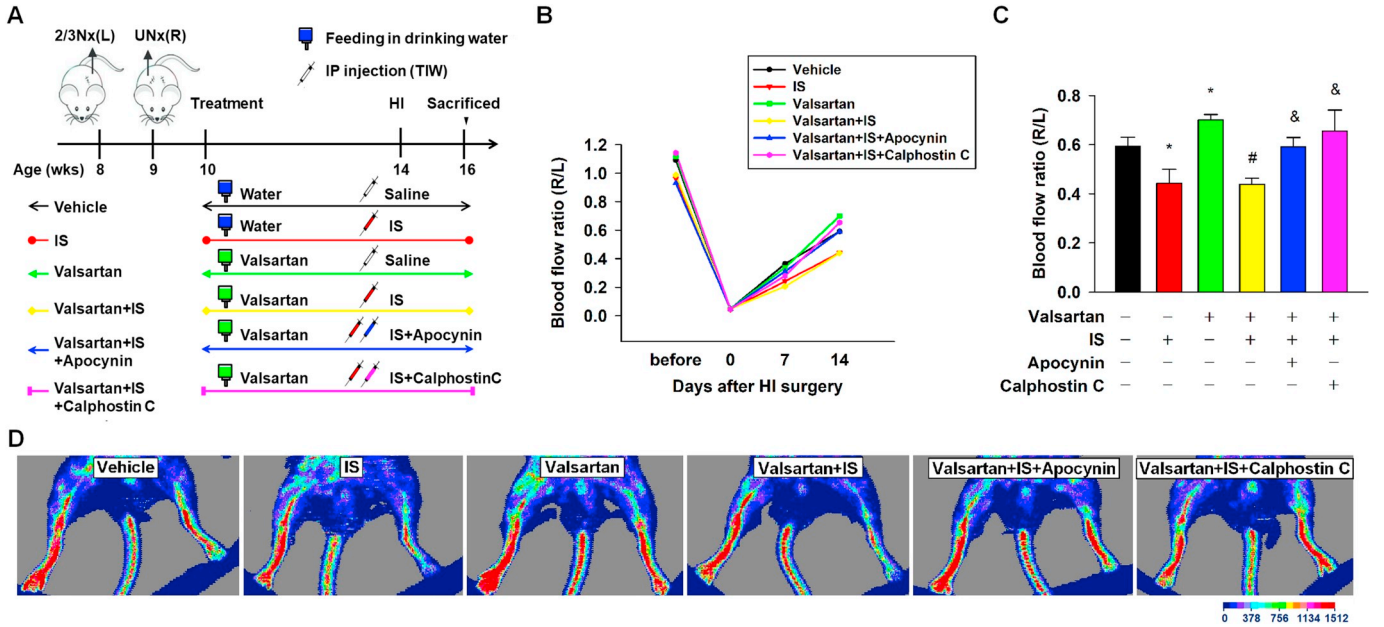

Fig. 6. Blood perfusion in the ischemic limbs of mice with subtotal nephrectomy after hindlimb ischemic surgery. (A) Protocol illustration of the effect of IS on neovascularization in SNx mice. Uremia was induced in 8-week-old male C57BL/6 mice using two-step subtotal nephrectomy (SNx). One week after subtotal nephrectomy, six SNx mouse groups receiving vehicle or medications in the following six weeks were created as follows: mice were administered with vehicle, mice were administered with valsartan (60 mg/kg/day in drinking water), mice were administered with IS intraperitoneally (IP, 100 mg/kg thrice a week), mice were administered with IS + valsartan, mice were administered with IS + valsartan + IP calphostin C (10 μg/kg thrice a week), mice were administered with IS + valsartan + IP apocynin (50 mg/kg thrice a week). (B) Blood perfusion in the ischemic hindlimb was measured before, immediately after, and at 1 and 2 weeks after hindlimb ischemic (HI) surgery by laser Doppler. (C) Quantification and (D) representative images of blood perfusion in the ischemic hindlimb was measured at 2 weeks after HI surgery by laser Doppler. Data are presented as the mean ± SEM from 8 mice. $^{*}P < 0.05$ vs. vehicle-treated mice. $^{\#}P < 0.05$ vs. valsartan-treated mice, $^{\&}P < 0.05$ vs. IS + valsartan-treated mice.

calphostin C or apocynin abrogated the detrimental effect of IS on blood perfusion induced by valsartan in ischemic limbs (Fig. 6B–D). These findings were consistent with the abovementioned Matrigel plug assay results.

3.7. IS limits the valsartan-induced neovascularization in the ischemic limbs of SNx mice after HI surgery

We further demonstrated that valsartan significantly improved EPC mobilization in circulation and the density of $CD31^{+}$ capillaries in the ischemic limbs of SNx mice (Fig. 7A and B). However, IS diminished the protective effect of valsartan-induced neovascularization and increased the expression of p-PKCα^{Ser657} and p-eNOS^{Thr497} in ischemic limbs (Fig. 7C). Cotreatment of calphostin C or apocynin reversed the IS-impaired neovascularization induced by valsartan and decreased the expression of p-PKCα^{Ser657} and p-eNOS^{Thr497} in ischemic limbs (Fig. 7A and C). These findings suggest that the NADPH oxidase-ROS-PKC signaling pathway may play a pivotal role in the IS-mediated inhibition of valsartan-conferred beneficial effects on endothelial function *in vivo*. A schematic illustration of the proposed mechanistic inhibition of IS on valsartan-induced neovascularization via NADPH oxidase and the PKC signaling pathway in SNx mice is shown in Fig. 7D.

4. Discussion

Endothelium-derived NO plays a pivotal role in the physiological functions of endothelial cells and endothelial progenitor cells [22,28,31]. NO bioavailability is tightly controlled by the enzymatic activity of eNOS [32]. Several studies have shown that increasing the NO bioavailability using pharmacological agents may be a therapeutic strategy for treating eNOS-related human diseases [23,33,34]. Valsartan abolishes the action of angiotensin II on AT1R, which exerts various antihypertensive effects, including relaxation of vascular smooth muscle cells (VSMCs) and inhibition of VSMC proliferation [35,36]. In addition, valsartan possesses pleiotropic activities, including antiathergenic, antiinflammatory, antiplatelet aggregation, and antithrombotic actions [37–39], all of which are linked to the bioavailability of NO. Furthermore, our previous study further disclosed that valsartan induces phosphorylation of eNOS, accompanied by dissociation of eNOS and AT_1R, which consequently increases NO production via the Src/PI3K/Akt signaling pathway [23].

Deregulation of eNOS activity is a key event in the development of hypertension and impaired angiogenesis in ischemic tissue diseases [40,41]. In addition to transcriptional regulation, posttranslational modifications are a crucial mechanism for regulating eNOS activity in response to various stimuli [32,34,41,42]. Among these stimuli, kinase-dependent phosphorylation at multiple eNOS sites is a pivotal mechanism for modulating eNOS activity. Phosphorylation of eNOS at both Ser1177 and Ser633 in human endothelial cells (Ser1179 and Ser635 in bovine endothelial cells) by Akt, calmodulin-dependent protein kinase II and AMP-activated protein kinase increases eNOS activity [34,42]. In contrast, phosphorylation of eNOS at Thr495 in human endothelial cells (Thr497 in bovine endothelial cells) by PKC decreases eNOS activity [34,42]. In our study, we found that IS increased the phosphorylation of eNOS at Thr495 rather than at Ser615, Ser633 and Ser1177 in HAECs. Moreover, IS attenuated valsartan-mediated NO production and tube formation in HAECs via increasing NADPH oxidase and PKCα phosphorylation.

Indole is produced by intestinal bacteria as a degradation product of the amino acid, tryptophan and is subsequently absorbed and metabolized in the liver to IS, the prototype of protein-bound uremic toxins. Studies have reported the detrimental effects of IS accumulation, such as increased ROS production, upregulation of NADPH oxidase, and reduced NO bioavailability [5–7]. Several clinical studies support the idea that IS may contribute to CVD in CKD and end-stage renal disease. Barreto et al. demonstrated that the serum levels of IS were positively

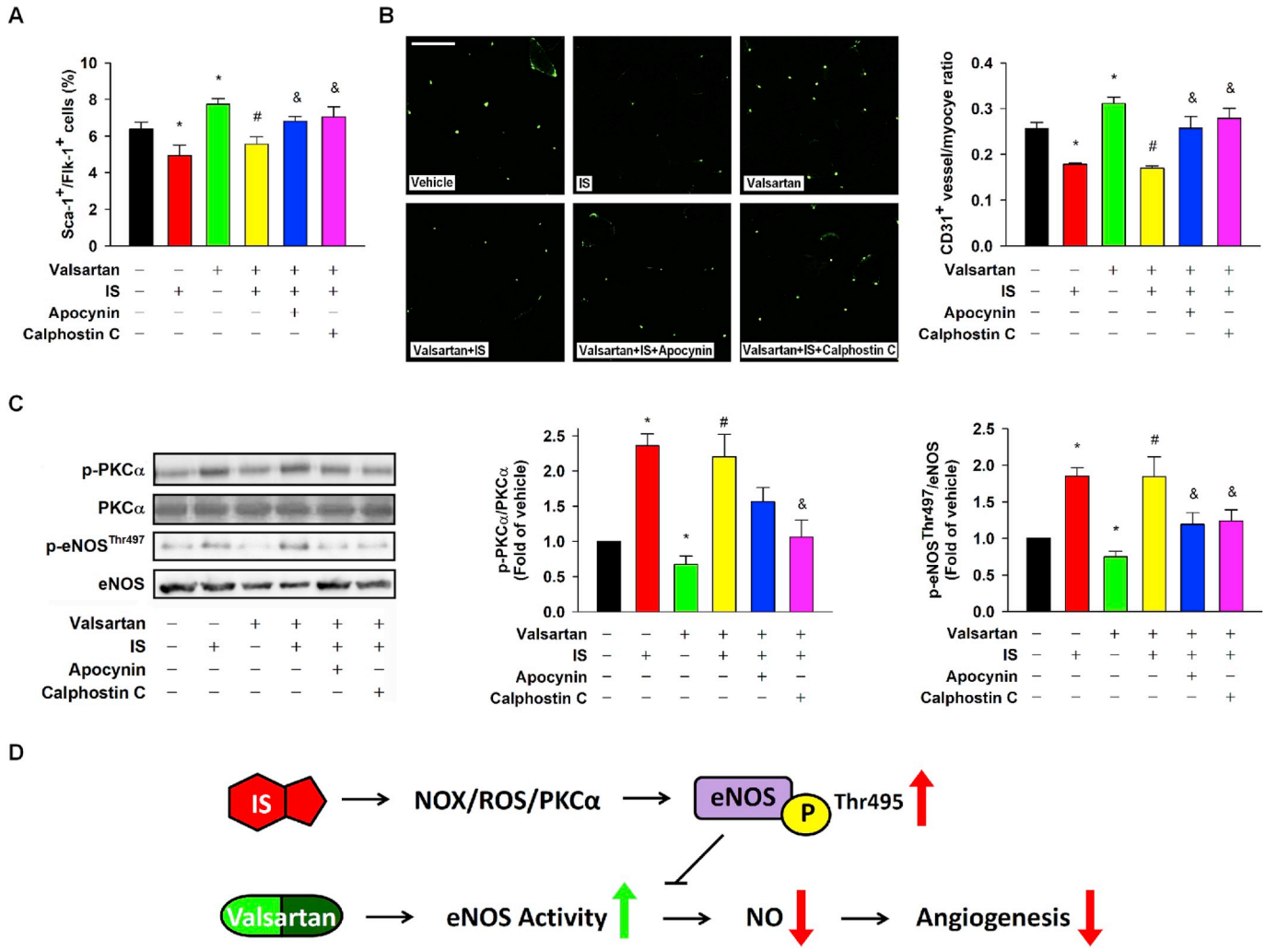

Fig. 7. IS impairs valsartan-induced neovascularization in the ischemic limbs of SNx mice after HI surgery. (A) Circulating Sca-1$^+$/Flk-1$^+$ cells were analyzed by flow cytometry at baseline and at 2 days after HI surgery. (B) Representative images and quantification of CD31 positive capillaries in the ischemic muscles. Scale bar = 50 μm. (C) Verification and quantification of phosphor-PKCα^{Ser657} (p-PKCα^{Ser657}), phosphor-eNOS^{Thr497} (p-eNOS^{Thr497}), PKCα and eNOS in the ischemic hindlimbs by Western blot analysis. (D) Schematic illustration of the proposed mechanism by which IS inhibits valsartan-induced neovascularization via the NADPH oxidase (NOX) and PKC signaling pathways in SNx mice. The results are presented as the means ± SEM. $^*P < 0.05$ vs. vehicle-treated mice. $^{\#}P < 0.05$ vs. valsartan-treated mice, $^{\&}P < 0.05$ vs. IS + valsartan-treated mice.

and significantly associated with aortic calcification and pulse wave velocity in patients at different stages of CKD [8]. Moreover, the highest IS tertile was a powerful predictor of overall and cardiovascular mortality [8]. Additionally, Lin et al. determined that the serum IS level was a valuable marker for predicting CV events in patients with advanced CKD [43].

Until now, evidence from experimental studies demonstrated that endothelial dysfunction [7,44–46], smooth muscle cell lesions [47,48], coagulation disturbances [47], leukocyte activation [45], cross-talk between leukocytes and the endothelium [45], cardiac fibrosis and hypertrophy [49], and whole-vessel alterations [50] are all linked to IS and cardiovascular damage. However, the direct cross-link between IS and ARB signaling in eNOS activity and further cardiovascular damage has never been reported. This hypothesis was first validated by our *in vitro* experiments (Figs. 1 and 2). We found that IS impaired the valsartan-mediated phosphorylation of eNOS at Thr495, NO production and tube formation in HAECs via NADPH oxidase and PKC phosphorylation, but these effects were suppressed by cotreatment with apocynin (NADPH oxidase inhibitor) and calphostin C (PKC inhibitor). Moreover, using *in vivo* experiments, we also demonstrated that IS attenuated valsartan-induced angiogenesis in Matrigel plugs in mice. Finally, to mimic the clinical condition in CKD, we used subtotal nephrectomized mice to validate the effect of IS on ischemia-mediated neovascularization. We demonstrated that valsartan significantly improved the EPC mobilization in circulation and the reperfusion of blood flow and density of CD31$^+$ capillaries in the ischemic limbs of CKD mice. However, IS attenuated the protective effect of valsartan-induced neovascularization and increased the expression of p-PKCα^{Ser657} and p-eNOS^{Thr497} in the ischemic limbs of CKD mice. Cotreatment with apocynin and calphostin C reversed the IS impaired-neovascularization and decreased the expression of p-PKCα^{Ser657} and p-eNOS^{Thr497} in the ischemic limbs of CKD mice. To our knowledge, this study is the first to show that IS impairs the protective effect of valsartan-mediated endothelial function in HAECs and neovascularization in CKD mice via the NADPH oxidase and PKC signaling pathways. We demonstrated that IS in not only a direct vascular toxin but also plays an important role in uremic toxin and drug interactions. Our study supports the meta-analysis study by Tai et al. [18] and that the use of RAAS blockades is not associated with a statistically significant reduction in the risk of CV events in CKD patients, unlike in the general population. Our results also provide another explanation for the high CV risk in CKD patients.

From a clinical viewpoint, several issues merit discussion in this

study. First, IS concentrations of 60–240 μg/mL in our *in vitro* study were within a higher but clinically achievable range of the plasma IS concentrations in dialysis patients [29]. Duranton et al. have used the similar IS concentrations in previous *in vitro* experiments [30]. Second, most IS binds to albumin in the blood. The culture medium for HAEC in our *in vitro* experiments contained 5% FBS, and the calculated albumin concentration was approximately 85–170 mg/dl. This concentration indicates that the medium in *in vitro* experiments was not albumin-free and mimicked *in vivo* conditions. Our data provide insight into the molecular mechanisms underlying the IS-mediated inhibition of valsartan-conferred beneficial effects in normal physiological situations, and an increase in IS levels may predispose mice to more down-regulated valsartan-mediated endothelial function and impair the promising effect of valsartan-mediated neovascularization in CKD. Third, intriguingly, although IS interfered with eNOS activity in our *in vitro and in vivo* studies, the increased IS levels did not further increase the blood pressures of CKD mice. This finding indicates that the adverse effect of IS on the cardiovascular system was BP-independent. Finally, since our study demonstrated that IS can constrain the cardiovascular protective effect of valsartan *in vivo* and *in vitro*, the current and our previous findings are important to support the increasingly recognized phenomenon that IS is not merely a cardiovascular toxin but also functions in toxin-drug interactions. Interventions aimed at preventing indole absorption and IS production can be achieved by three approaches, including dietary protein restriction, maintenance of gut symbiosis, and treatment with Kremezin (Kureha Chemical Industry, Tokyo, Japan), an oral charcoal adsorbent of uremic toxins [51]. Encouraging results, both decreased IS levels by treating with Kremezin and CV protective effects, have been shown in patients with CKD from some clinical studies [46,52–54].

In conclusion, the results of *in vitro* experiments demonstrated that the NADPH oxidase/PKC/eNOS phosphorylation signaling pathway plays a pivotal role in the IS-mediated inhibition of valsartan-conferred beneficial effects on endothelial function. In addition, IS limited the valsartan-elicited angiogenesis in a Matrigel plug assay in mice. Finally, we also demonstrated that IS attenuated valsartan-mediated neovascularization in SNx mice who underwent hindlimb ischemia. We herein demonstrate novel mechanisms by which IS detrimentally interferes with the therapeutic efficacy of valsartan in CKD. Likewise, our results together with those of our previous study [27] provide compelling evidence of potential adverse CV outcomes for CKD patients with high plasma IS levels. Oral charcoal sorbents may potentially offer a therapeutic rationale for the prevention and treatment of impaired neovascularization in CKD patients with cardiovascular disease.

Author contributions

K.L.K., D.C.T and T.S.L. designed the study; K.L.K, P.H.H. and J.F.Z. carried out experiments; K.L.K, and J.F.Z. analyzed the data; K.L.K, and J.F.Z. made the figures; K.L.K., T.S.L., and D.C.T. drafted and revised the paper; all authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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